

MERISTEM CULTURE OF MINIATURE MONOPODIAL ORCHIDS

A THESIS SUBMITTED TO THE GRADUATE DIVISION OF THE
UNIVERSITY OF HAWAII IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

IN HORTICULTURE

MAY 1972

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ACKNOWLEDGEMENT

The author expresses deep appreciation to the East-West Center for the scholarship to pursue graduate work at the University of Hawaii, the American Orchid Society for partial financial support for equipment, and to Christopher Teo for technical assistance in writing.

INTRODUCTION

The shoot-tip (meristem) culture technique has been widely used for the clonal propagation of orchids. This method is probably the biggest breakthrough in orchid propagation since the early 1920s, when the method of asymbiotic seed germination was reported by Knudson (1922). Propagation by shoot-tip culture makes possible the production of numerous identical plants from commercially desirable hybrids which are generally extremely heterozygous within a short period of time. Current asexual methods of propagation such as division of rhizomes or topping of the mother plant yield only a limited number of plants. Thus, they are slow and costly.

Meristem culture is now being used on a commercial scale with Cymbidium (Morel, 1960, 1964; Sagawa et al., 1966), and Cattleya (Reinert and Mohr, 1967; Scully, 1967; Lindemann, 1970). Other sympodial orchids which have been successfully propagated by meristem culture include Dendrobium, Calanthe, Odontonia, Vuylstekeara, Spathoglottis, Cryptopodium, Lycaste, Miltonia, Odontoglossum, Phaius, Paphiopedilum, Oncidium, and Zygopetalum (Morel, 1965, 1971; Vaucherot, 1966; Bertsch, 1967; Sagawa et al., 1967). The monopodial type of orchid like Phalaenopsis and Vanda does not or barely responds to the standard techniques (Scully, 1967). Vajrabhaya (1970) reported only limited success of Rhynchostylis gigantea in meristem culture.

The main purpose of this study was to find out whether miniature monopodial orchids could be propagated by meristem culture as successfully as sympodial orchids. In addition, if they did respond to

meristem culture, the following were sought:

- (1) to determine what sources of explants could most readily be induced to form protocorm-like bodies (plbs);
- (2) to study the growth pattern, and the effect of agitation on the differentiation of protocorm-like bodies into plantlets;
- (3) to find a suitable medium for proliferation, differentiation and growth of tissues up to the plantlet stage.

LITERATURE REVIEW

Since Morel (1960) demonstrated the usefulness of meristem (shoot-tip) culture as an asexual method of propagation as well as of eliminating virus from orchids, this technique has been discussed extensively among orchid growers. The various techniques for successful culture of shoot and leaf tips were described by various workers (Morel, 1960, 1964; Sagawa et al., 1966; Reinert and Mohr, 1967; Lindemann, 1970; Churchill et al., 1970, 1971). In the sympodial orchids like Cattleya, Cymbidium, Dendrobium, a young shoot arising from a "back bulb" or from a "green bulb" provided suitable material for a source of explants. Kim et al. (1970) showed that the best source of explant in Dendrobium came from those shoots weighing between 1-3 gm and having an average length of 5 cm. Explants from apical buds were most likely to form plbs. Vajrabhaya (1970) used axillary and terminal buds. Personal communication with Iwanaga and Taba Lab (2610 Waiomoa Rd., Honolulu 96816) indicated that newly initiated flower stalks (0.75 cm) can be used as a source of explants.

Wimber (1965) recognized that leaf tissue of some aseptically grown Cymbidium plantlets in liquid medium produced plbs when constantly buffeted against the sides of the glass flasks at 200 rpm. Churchill et al. (1970, 1971) were able to induce callus production from the leaf tips of seedlings and mature Cattleya, Laeliocattleya and Epidendrum plants. Only the young tissues obtained before the development of the notch so typical of Cattleya and Laeliocattleya leaf tips could form callus. Tissues removed after the notch was

evident did not produce callus. Attempts to induce callus from other parts of the leaf failed.

Liquid vs. Solid Media and Effect of Agitation

Tissues grow better in liquid cultures than on agar (Steward et al., 1952). Similarly, Reinert and Mohr (1967) showed that the growth of Cattleya explants was generally faster and callus development was more frequent in liquid medium than on agar. When placed directly on an agar medium during the first three weeks of growth, excised meristem tissues turned brown and subsequently died, whereas, tissues submerged in liquid culture grew and developed normally. Apparently the tissues receive enough oxygen through agitation of the culture. For Cymbidium and Dendrobium solid or liquid media served equally well (Sagawa and Kunisaki, 1968).

Wimber (1963) found that in a flask which was not agitated, the meristem tissue followed a developmental sequence which superficially resembled that of seed germination, i.e., after the protocorm had reached about 4 mm size, a shoot appeared. In flasks which were agitated, these plbs did not generally produce shoots but continued to proliferate into more plbs. It was considered that shaking apparently checked any polarity that might develop in the tissue and this inhibited shoot formation. Scully (1967) listed the following possible effects of agitation: (1) to dilute some specific inhibitor produced in response to excision and sterilization; (2) to improve aeration, ultimately providing for full activity in respiration, protein synthesis, and salt uptake; (3) or, in case of proliferating

tissues, to increase the surface area exposed to nutritive materials.

Nutrient Media

The requirements for tissues in culture are little different from the requirements of germinating seeds (Wimber, 1963; Morel, 1965). Simple inorganic nutrient media with sugar and coconut water were the most suitable general culture medium (Sagawa and Kunisaki, 1968). Morel (1965) found that the complex media containing plant extracts like banana, tomato juice or coconut water used for seedlings gave poor results, and Knudson C medium was preferred. There were, however, some workers (Lindemann, 1967; Vajrabhaya, 1970; Churchill et al., 1970, 1971) who showed that certain species of orchids needed more than just simple media in order for tissues to proliferate. Vajrabhaya (1970) formulated a medium for culturing tissue of Rhynchosstylis, a monopodial orchid, which responded poorly to standard techniques. Lindemann (1967) found that using "starting, maintenance and rooting" media for Cattleya culture gave better results than just simple, uniform nutrient media used throughout the stage from proliferation to plantlet. In culturing leaf tips, Churchill et al. (1970, 1971) used modified Murashige-Skoog and modified Heller's medium to obtain callus. To obtain plantlets from the callus and protocorms produced, Knudson C medium was used.

The mineral requirements of a given tissue is not fixed but depends on its physiological state. The biochemical-physiological differentiation in orchids appeared to parallel the more evident morphological-anatomical differentiation (Arditti, 1965). Thus, in

principle, the chemical composition of nutrient solution should be adapted to each particular growth stage, and even the size of tissue at a particular time. Requirements for growth factors vary according to the species, and even to the nature of the tissues from one and the same species.

Most plant tissues are not carbon autotrophic. Even if the initial explant is rich in chlorophyll, it becomes more or less completely depigmented during the culture, and photosynthesis ceases. The tissues must then be cultivated in sugar-containing media (Ueda and Torikata, 1968). Gautheret (1955) showed that sucrose was the best carbon source followed by glucose, maltose, raffinose, fructose and galactose. Galactose caused damage and retarded the growth of seedlings (Ernest et al., 1971). Pentoses and polysaccharides cannot be utilized by normal carrot tissue. Sucrose had been used in concentrations ranging from 2-5% for good growth. Yates and Curtis (1949) grew Cattleya on media containing 0.06 to 0.24 molar sucrose. As the plants became older (about one year old), increased root growth was conspicuous and shoot growth was notably decreased with higher concentration of sucrose. Ueda and Torikata (1968) showed that increasing the sucrose concentration of the media considerably increased the fresh weight and branching of Cymbidium protocorms, the most abundant growth being observed with 4% sucrose. According to Hayes (1969), although sugar was necessary for practical success, its requirement was not absolute. Arditti (1965) stated that sugar free media were incapable of supporting any appreciable growth regardless of additives.

The use of coconut water for in vitro culture was first proposed by van Overbeek et al. in 1941. The most effective concentrations vary from 5 to 25%. Addition of coconut water induced strong proliferation but retarded differentiation of Phalaenopsis seedlings (Ernest, 1967). When provided in the germination medium, coconut water inhibited the growth of Dendrobium seedlings but stimulation of growth was observed in one year old seedlings (Kotomori and Murashige, 1965).

Control of Differentiation

Root and shoot initiation from solid cultures (Murashige, 1965) and agitated liquid cultures (Bonnett and Torrey, 1965) could be controlled to some extent by changing the type of growth regulator used (Maheshwari and Gupta, 1965; Kent and Steward, 1965) or altering its concentration (Staba et al., 1965). Coconut water (Halperin, 1966b; Steward et al., 1964) and reduced nitrogen (Reinert et al., 1966; Halperin, 1966a) may or may not be needed to induce plantlets. The technique of inducing plantlets is sufficiently advanced so that thousands of similar Cymbidium plants were produced within a year from a meristem (Morel, 1964). The ability to regulate plbs so that they grow continuously, as either undifferentiated tissues (plbs) or as differentiated tissues (plantlets), is of scientific as well as commercial significance.

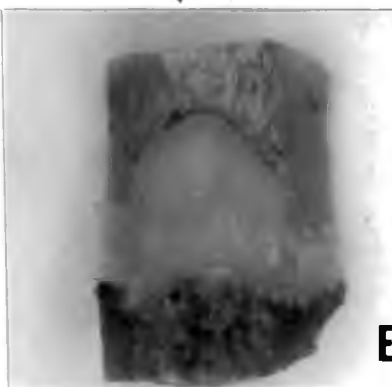
MATERIALS AND METHODS

The miniature monopodial orchids used in this study were obtained from the collection of the Horticulture Department, University of Hawaii. The following hybrids were used:

Name	Hybrid code number	Parents
<u>Ascofinetia</u> Twinkle	1800	<u>Neofinetia falcata</u> x <u>Ascocenda miniatum</u>
<u>Ascofinetia</u> Twinkle	1873	<u>Neofinetia falcata</u> x <u>Ascocenda miniatum</u>
<u>Ascofinetia</u> Cherry Blossum	1799	<u>Neofinetia falcata</u> x <u>Ascocenda ampullaceum</u>
<u>Vandofinetia</u> Premier	1908	<u>Neofinetia falcata</u> x <u>Vanda lamellata</u>
<u>Neostylis</u> Lou Sneary	1907	<u>Neofinetia falcata</u> x <u>Rhynchostylis coelestis</u>
<u>Neostylis</u> Lou Sneary	1924	<u>Neofinetia falcata</u> x <u>Rhynchostylis coelestis</u>
<u>Vascostylis</u> Blue Fairy	1923	<u>Ascocenda Meda Arold</u> x <u>Rhynchostylis coelestis</u>

Sources of explants used were from vegetative shoots of the six to seven leaf stage (Figs. 1A, B). The leaves of the shoots were removed (Fig. 1C) and the shoot axis was then sterilized for 15 minutes in 10% clorox with one drop of Tween-20 in 100 ml liquid. The leaf-bases still attached to the shoot axis were removed (Fig. 1D). The shoot axis was again placed in 5% clorox solution for 10 minutes. To remove traces of clorox, the sterilized materials were then soaked in sterile distilled water for three minutes.

Fig. 1. Method of obtaining explants. Plants used for meristem culture (A x 1/4). Vegetative shoot of seven-leaf stage, a suitable source of explant (B x 1/3). Shoot axis after leaves were removed (C x 1/2). Axis of stem with terminal and axillary buds (D x 1). An explant from an axillary bud (E x 10).



Two kinds of buds were used as explants: (1) the axillary buds, which are located in the axil of the leaves. Usually three to seven buds were found on a stem. They are almost entirely covered by flat scales (Fig. 1D). The size of the buds was approximately 2-3 mm (Fig. 1E). The buds in the detached shoots were numbered basipetally. (2) the terminal buds, which consisted of the apical meristem plus two to four leaf primordia.

Both axillary and terminal buds were cultured in vials (4" x 1") on Vacin and Went medium (1949) modified by the addition of 15% coconut water (v/v). For solid medium 9% agar (w/v) was added.

Cultures were placed under 200 foot-candle continuous illumination, obtained from G.E. white fluorescent lamps (Power Groove). The temperature was $26 \pm 3^{\circ}\text{C}$.

After one or two months of culture on agar medium, the scale-like leaves which enclosed the growing tissue were removed and the tissues were transferred into liquid medium. The 50 ml erlenmeyer flask containing 20 ml of medium was continuously agitated at approximately 160 rpm using a New Brunswick Model V shaker. After four to five weeks in the agitated medium, plbs began to form. In some cases, removal of scale-like leaves did not result in proliferation and scale leaves developed instead. These needed to be removed again to induce proliferation.

Inflorescences with floral primordia at various stages of differentiation were removed from the shoots and measured for length for inflorescence culture. Sterilization was done as described earlier. After the bracts were removed, these inflorescences were cultured in

agitated liquid medium.

Once plbs were obtained, the amount of sucrose in the medium was reduced to zero. Otherwise the plbs turned yellow. Plbs were subcultured into fresh liquid medium every two to three weeks.

For anatomical study, tissues were killed and fixed in Craf fixative, dehydrated in a series of tertiary butyl alcohol, and embedded in paraffin. Sections cut 10 to 12 μ thick were stained by the rapid Safranin and Fast Green technique (Shapiro, 1947). For morphological and anatomical observations and photography, a Wild M-5 stereo-microscope or Zeiss photomicroscope was used.

RESULTS

1. Study on the ability of different hybrids and buds from different positions to form plbs

A total of 136 explants were cultured and 53 (39.0%) of these were contaminated with fungus or bacteria. Out of the 83 uncontaminated cultures, 26 (31.3%) proliferated within 58 - 118 days and produced plbs. The percentage of cultures that formed plbs varied from 16.0 - 66.7% depending on the hybrids (Table I). The highest percentage of success in plb formation was obtained with hybrid 1873, while the lowest was with hybrid 1907.

As shown in Table I explants of apical buds resulted in 66.7% plb formation. Axillary buds from positions one to four resulted in 33.3 to 20.0% plb formation. The buds from positions five and below did not proliferate.

In inflorescence culture, inflorescences of $1\frac{1}{2}$ cm or less proliferated (Fig. 2A) within 20 to 51 days (Table II), whereas those which were three cm or longer either had elongated internodes and florets which turned brown (Fig. 2B) or florets which developed and bloomed (Fig. 2C).

TABLE I. The ability of different hybrids and buds from different positions to form protocorm-like bodies

Sources	Hybrid number						Total	Percent
	1800	1873	1908	1907	1924	1923		
Apical buds	2/4 ^b (58)	1/1 (68)	3/3 (107)	0/1	0/1	2/2	8/12	66.7
Axillary buds								
Position 1 ^a	0/2	1/3 (96)	1/4 (98)	2/7 (118)	1/4 (61)	3/4 (90)	8/24	33.3
Position 2	0/1	0/0	1/4 (60)	0/3	1/4 (114)	2/4 (107)	4/16	25.0
Position 3	0/1	2/2 (104)	0/1	1/6 (107)	0/3	1/3 (103)	4/16	25.0
Position 4	0/0	0/0	0/1	1/6 (114)	1/3 (61)	0/0	2/10	20.0
Position 5	0/0	0/0	0/0	0/1	0/1	0/0	0/2	0
Position 6	0/0	0/0	0/0	0/1	0/1	0/0	0/2	0
Position 7	0/0	0/0	0/0	0/0	0/1	0/0	0/1	0
Total	2/8	4/6	5/13	4/25	3/18	8/13	26/83	
Percent	25.0	66.7	38.5	16.0	16.7	61.5		31.3

a The axillary buds are numbered basipetally

b Numbers of cultures forming plbs/total number of uncontaminated cultures

() Average number of days to proliferate

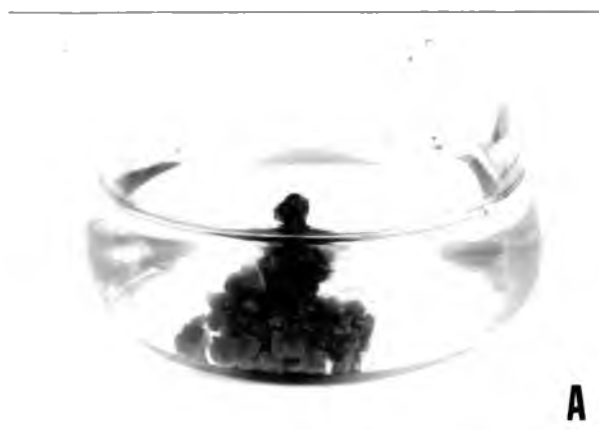
TABLE II. Proliferation of tissues from young inflorescences

Hybrid number	Inflorescence number	Inflorescence length (cm)	Proliferation	Days to proliferate
1923	1	3.0	-	-
1907	1	3.5	-	-
1799	1	1.0	+	34
1799	2	0.7	-	-
1799	3	1.0	+	37
1799	4	1.4	+	37
1799	5	1.0	+	47
1799	6	1.0	+	51
1799	7	0.8	+	40
1799	8	1.2	+	34
1799	9	1.0	+	34
1799	10	1.0	+	20
1799	11	1.0	+	34
1799	12	1.1	+	37

- no proliferation

+ proliferation

Fig. 2. Inflorescence culture. Proliferation of tissue from young inflorescence (A x 1.2). Unsuccessful culture of young inflorescence. Pedicels were elongated and florets turned brown and died (B x 1.2). Unsuccessful culture of young inflorescence in which florets developed and bloomed (C x 2.5).



2. Growth pattern of explants and differentiation into plantlets

A. Explants from axillary and terminal buds

The explants increased in size for one to two months in culture on solid VW medium without tissue proliferation. Scale-like leaves were visible surrounding the apical meristem. If these scale-like leaves were not removed from explants of axillary buds a shoot developed (Fig. 3A), while several shoots developed from the terminal bud explant (Fig. 3B). However, proliferation of tissues into plbs started four to five weeks after removal of these scale-like leaves and culture in agitated liquid medium (modified VW).

Plbs were observed to originate from various points on the explant.

(1) Tissues proliferated from the cut surface of the scale-like leaves at the nodal area (Fig. 4A).

(2) Plbs were also formed from the surfaces of the young leaves (Fig. 4B).

Usually proliferation was acropetal on the explant. Most of the initial explants which did not proliferate turned brown and degenerated slowly. The initially clear medium turned turbid to brown with time. If the medium was not changed after 10 to 14 days, growth of plbs slowed down.

The pattern of explant development can be classified in one of the following three ways:

(1) Explant grew directly into shoots without forming plbs (Fig. 5A).

- (2) Explant produced both one or more shoots and plbs (Fig. 5B).
- (3) Explant produced plbs only (Fig. 5C).

Growth of plbs started slowly and increased rapidly with time (Fig. 6). Hybrid 1923 produced the highest fresh weight of plbs while hybrid 1873 the least. Plants within the same hybrid also differed in the fresh weight of plbs produced as illustrated by hybrids 1924 and 1907.

Initially, plbs did not differentiate organs but kept on multiplying (Fig. 7A), several protuberances were formed and each green plb produced a number of similar bodies which remained attached to each other. However, leaves were visible 75 to 100 days after proliferation (Fig. 6). At this stage, the culture was still in agitated liquid medium (Fig. 7B). As more leaves began to develop the movement of plbs in the agitated medium became restricted due to crowding within the flask and, therefore, could be considered stationary (Fig. 7C). In hybrids 1923 and 1924, roots appeared 50 to 60 days after the tissue became stationary (Fig. 8A). Root hairs formed on roots of plbs that remained above the liquid medium surface (Fig. 8B). In hybrids 1908, 1907 and 1873, no roots were observed (Fig. 6).

To encourage the growth of more plbs, fragments of plbs were cultured in agitated medium. As long as the tissues were subcultured every two weeks and flasks kept agitated and not congested, multiplication of plbs continued.

A plb consisted of a mass of undifferentiated parenchyma cells. Several meristematic areas were observed within this mass (Fig. 9). These meristematic areas consisted of isodiametric cells which had

dense cytoplasm with prominent nuclei. From these cells originated hemispherical protuberances and leaf primordia (Fig. 10). No vascular system was observed in the plbs in the early stage prior to differentiation of leaves (Fig. 9). However, after leaf formation, paraffin sections of plbs revealed the presence of xylem elements (Fig. 11).

Fig. 3. Buds grown in solid Vacin and Went for 1-2 months.

Axillary bud from which a shoot developed (A x 15). Terminal bud from which several shoots developed (B x 15).



Fig. 4. Morphological origin of protocorm-like bodies. Tissues proliferated at the cut surface of the scale-like leaves at the nodal area (A x 10) and from the surfaces of the young leaves (B x 10).

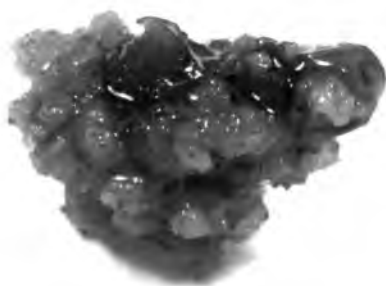
**A****B**

Fig. 5. Pattern of explant development. Explant grew directly into shoots without forming protocorm-like bodies (plbs) (A x 10), explant produced both shoot and plbs (B x 10), and explant producing plbs only (C x 10).



Fig. 6. Growth of protocorm-like bodies (plbs) from terminal and axillary buds in agitated liquid medium.

L=Leaves visible

S=Stationary plbs in agitated media

R=Roots visible

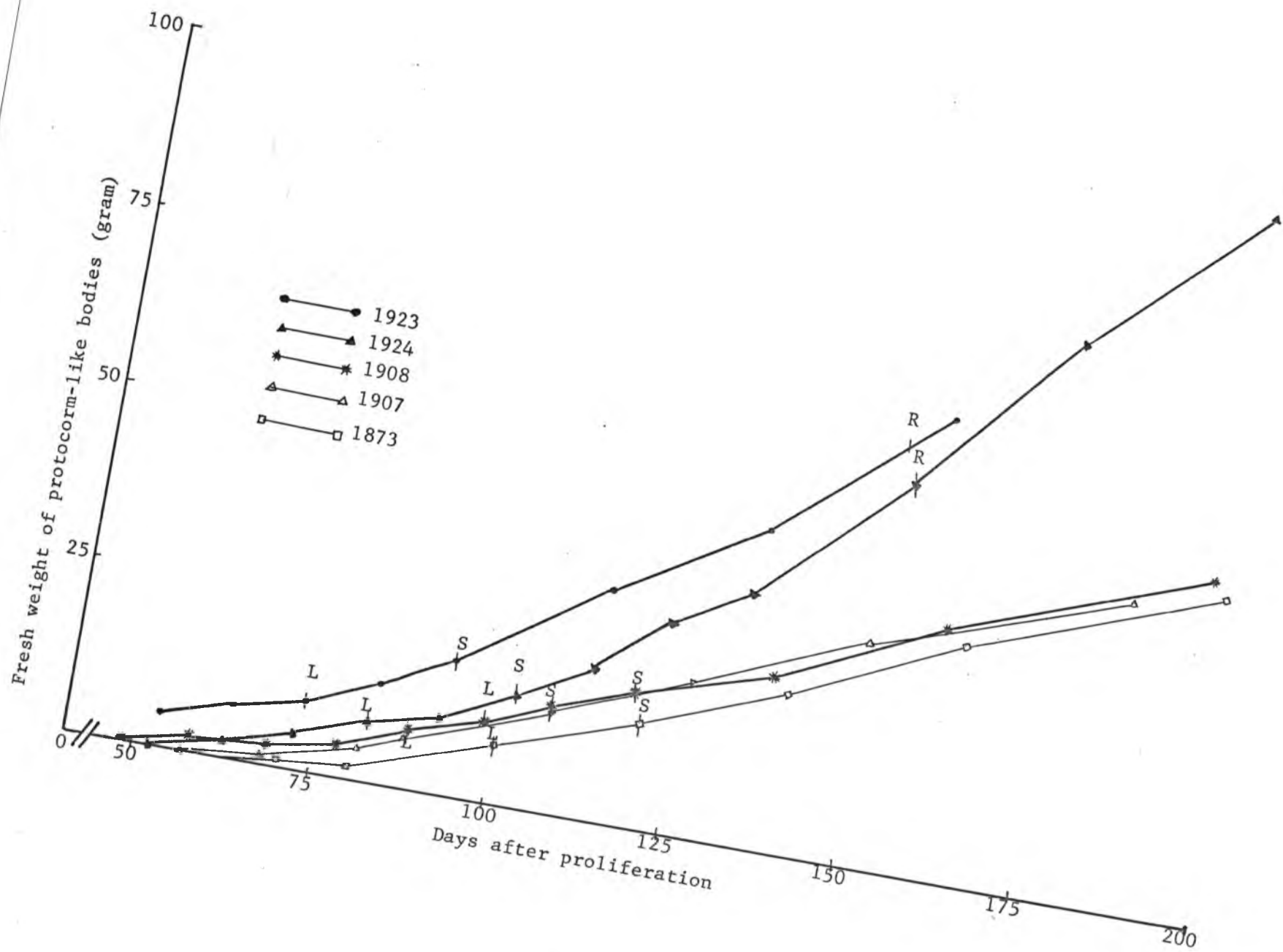


Fig. 7. Growth of protocorm-like bodies (plbs) in agitated liquid media. The non-differentiated rapidly multiplying plbs (A x 1.2), plbs with differentiated leaves (B x 1.2), and plbs with differentiated leaves and roots (C x 1.2).

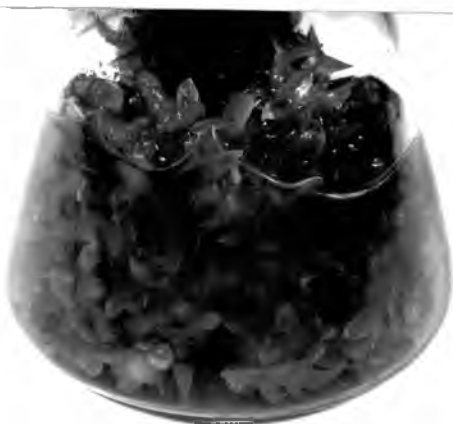
**A****B****C**

Fig. 8. Plantlets from protocorm-like bodies (plbs) grown in agitated liquid medium. Plbs differentiated into roots and leaves (A x 6), root hairs were formed on roots that remained on liquid surface (B x 6).

**A****B**

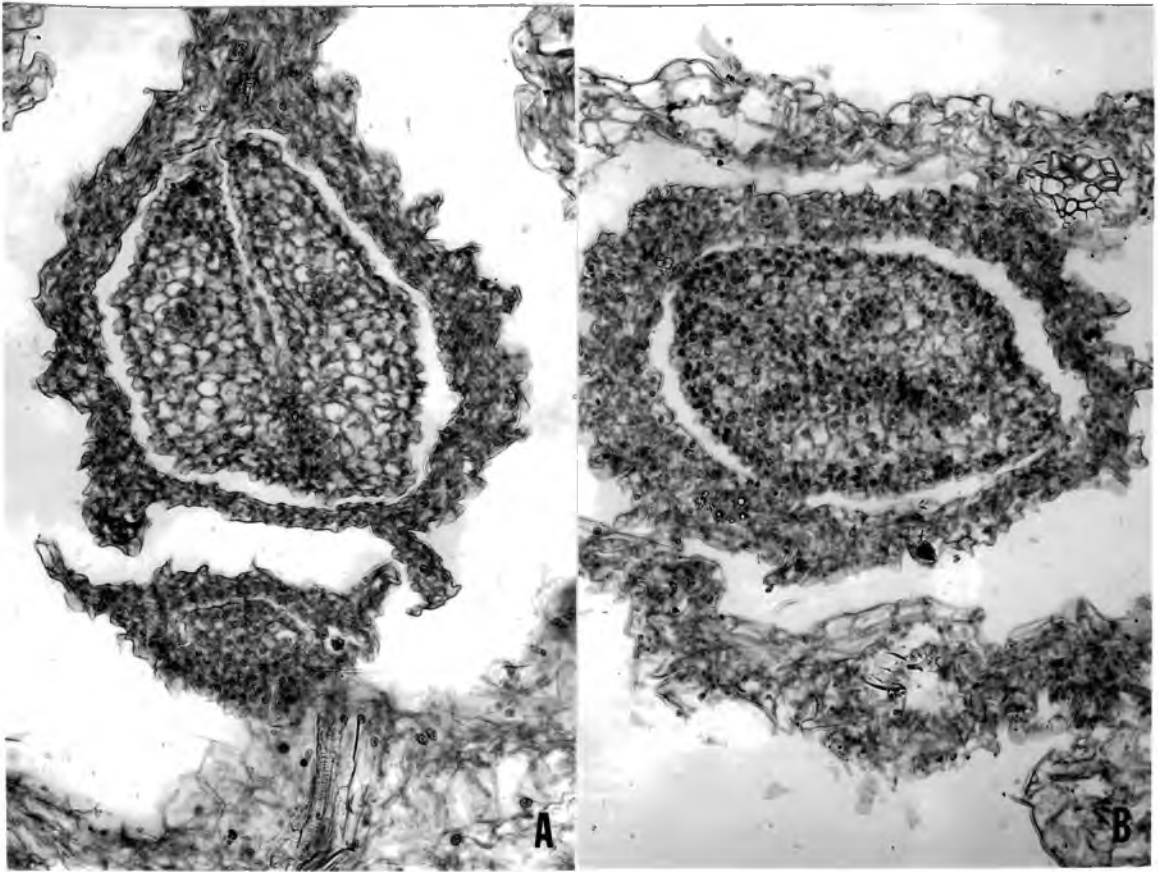
Fig. 9. Anatomy of protocorm-like bodies showing meristematic areas within the tissue mass (x 60).



Fig. 10. Anatomy of protocorm-like bodies showing leaf primordia derived from cells of the meristematic area (x 200).



Fig. 11. Longitudinal (A) and cross sections (B) of protocorm-like bodies showing the presence of xylem elements (x 200).



B. Explants from young inflorescence

Paraffin sections 10 to 12 μ were made from young inflorescences one week after proliferation (Figs. 12, 13). A layer of meristematic cells, derived from the epidermis, formed calli from which plbs developed. The parenchymatous cells of the cortex and pith did not show sign of meristematic activity, and numerous vascular bundles were developed. In the region where the bract was removed, no callus was formed.

Fig. 14 showed that at the base of the proliferating plbs a necrotic layer was formed from the outer cortical cells of the original tissue. This necrotic layer caused the separation of plbs from the original explant. Subsequent multiplication of plbs was from the newly formed plbs. The original tissues did not proliferate further and turned brown. Tissues from the bracts (Fig. 15A) and florets (Fig. 15B) on the young inflorescence did not proliferate and eventually turned brown.

The rate of growth of plbs from inflorescence culture is shown in Fig. 16. Initially growth was slow but after 50 days rapid growth ensued. Leaves appeared 86 days after proliferation. The flask began to get congested and within 20 days, the culture became stationary. Two months later, roots were formed.

Fig. 12. Longitudinal section of young inflorescence one week after proliferation showing layers of meristematic cells derived from the epidermis. The parenchymatous cells of the cortex and pith did not show sign of meristematic activity (x 60).

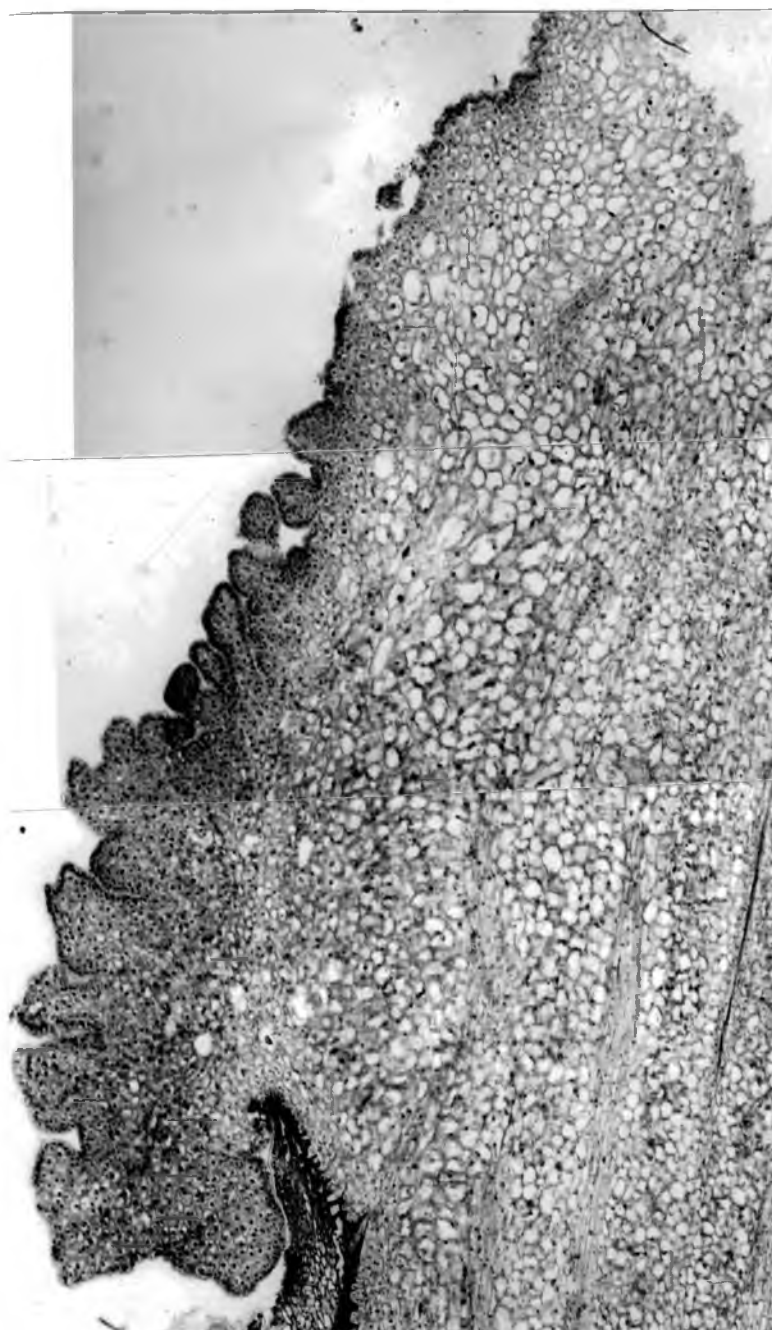


Fig. 13. Cross section of inflorescence one week after proliferation, showing meristematic cells formed on epidermis all over the surface of peduncle (x 60).

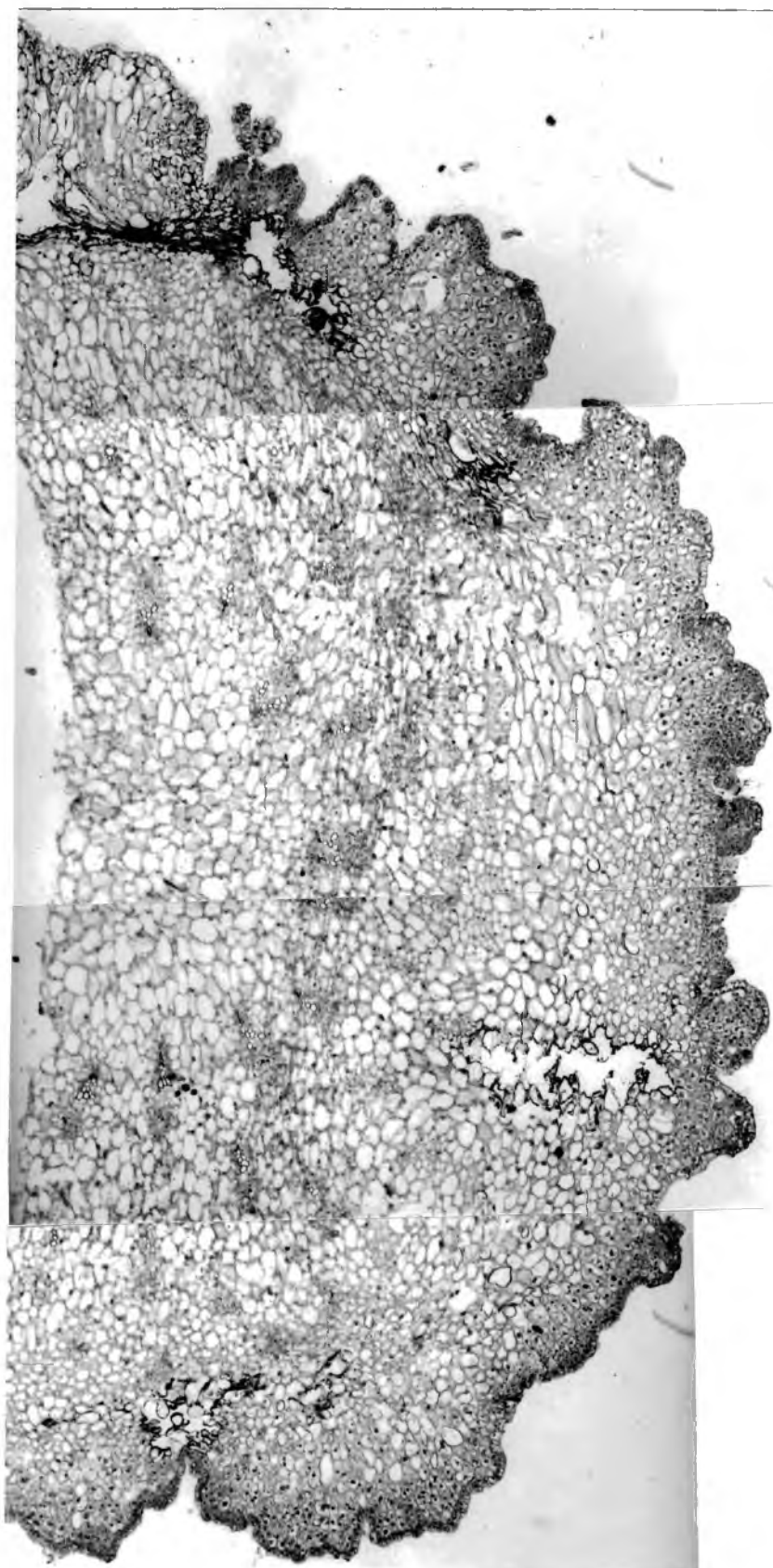


Fig. 14. Cross section of older portion of inflorescence, showing layers of necrotic cells which formed on the outer cortex of peduncle, caused the separation of protocorm-like bodies from the original explant (x 60).

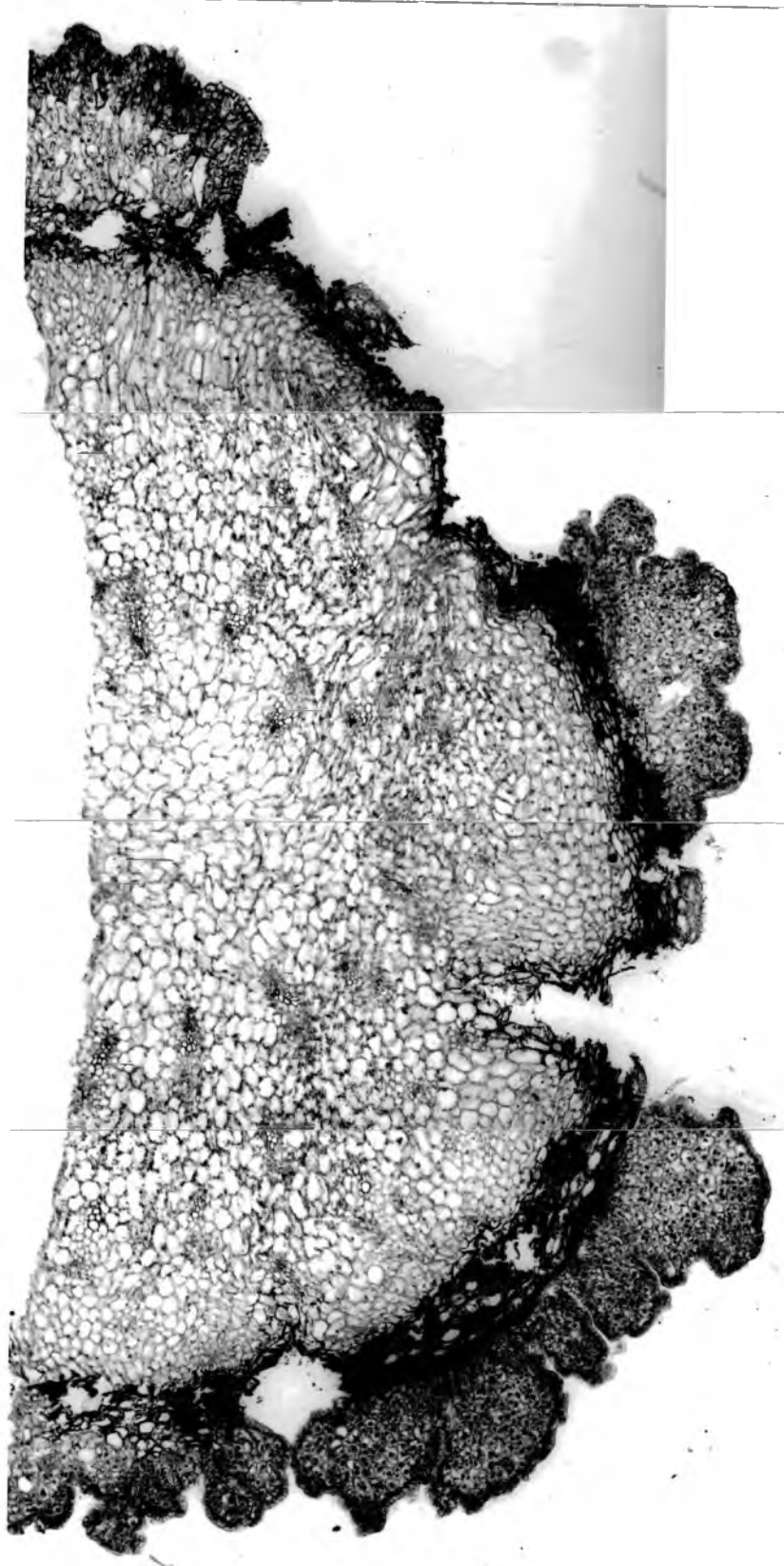


Fig. 15. Young inflorescence culture showing tissue from bracts (A x 1.2) and florets (B x 1.2) did not proliferate. Protocorm-like bodies were formed from tissues at the base of inflorescence.

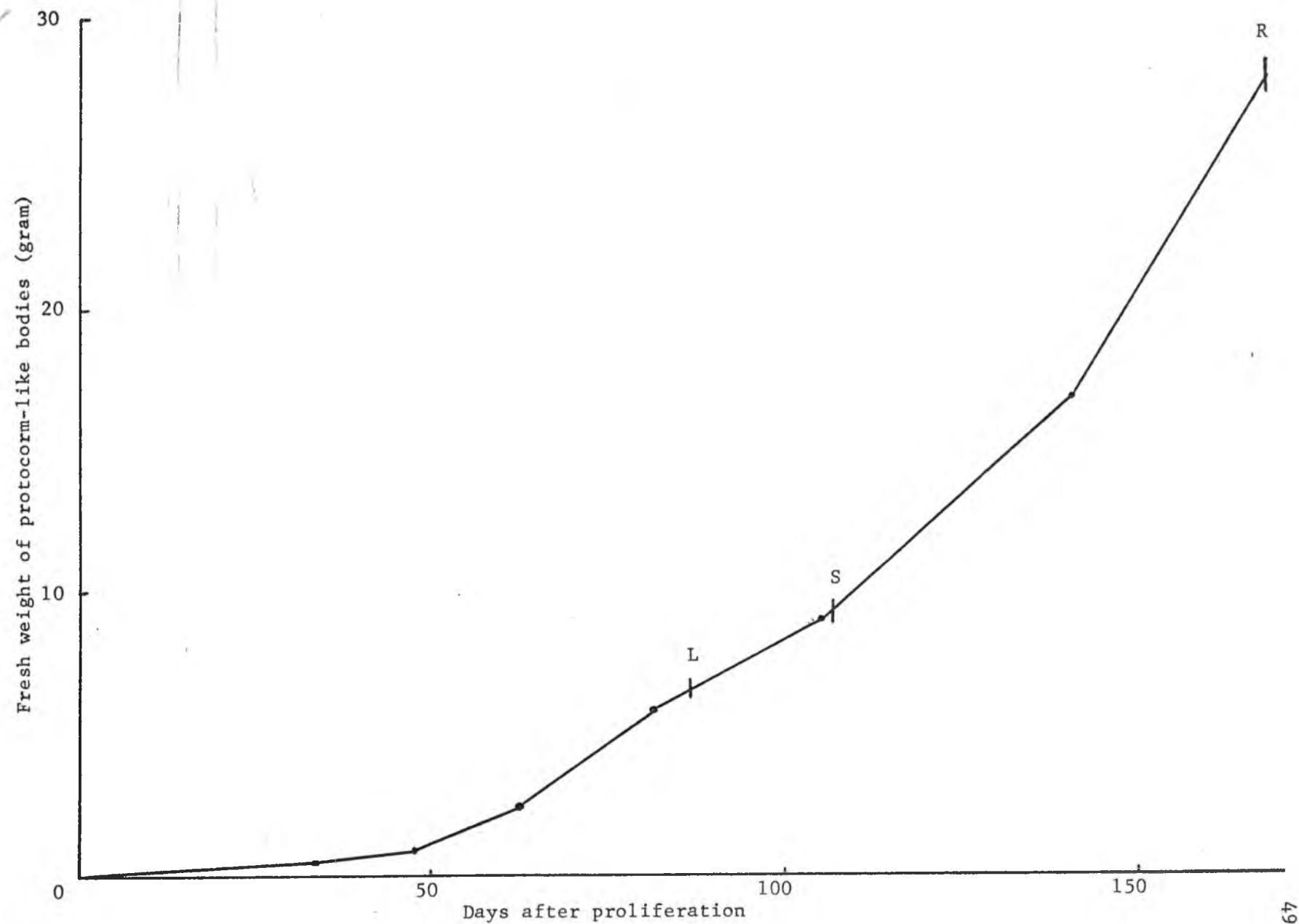


Fig. 16. Growth of protocorm-like bodies (plbs) from young inflorescence in agitated liquid medium.

L = Leaves visible

S = Stationary plbs in agitating media

R = Roots visible



3. Study of various media suitable for plb growth into plantlets

One-leaf stage plbs (Fig. 17) were transferred from the agitated liquid to the following solid media:

- (1) modified VW
- (2) modified VW without sucrose
- (3) VW
- (4) VW without sucrose

The two-month old plb cultures are illustrated in Fig. 18A. Modified VW without sucrose medium resulted in the greatest multiplication of plbs and some plbs had two to more leaves. However, on this medium no roots were produced, but there were numerous rhizoids (Fig. 19A). On modified VW, plbs proliferated but eventually turned yellow to brown. On VW most of the plbs turned brown, although some grew into plantlets and produced roots (Fig. 19B). There was little proliferation and differentiation of plbs when grown on VW without sucrose. When cultures which were yellow were transferred to modified VW without sucrose, they became green and resumed growth.

Two to three-leaf stage plbs from modified VW without sucrose medium were transferred to the four similar media list previously (Fig. 18B). After three months plbs on modified VW turned brown. On modified VW without sucrose, most tissues proliferated into plbs, but only seven plantlets were obtained. Most plbs cultures on VW turned brown, but some plbs differentiated into plantlets. A total of 25 plantlets were obtained. Plbs grown on VW without sucrose were yellow with little proliferation and differentiation.

The plantlets from the VW medium were grown on the same four different media listed earlier. On modified VW, plantlets grew very rapidly and were $1\frac{1}{2}$ inches high three months later (Fig. 16C). Plantlets on VW without sucrose showed the slowest growth and some died three months later.

Fig. 17. One leaf-stage of protocorm-like bodies used for culture on solid medium (x 6).

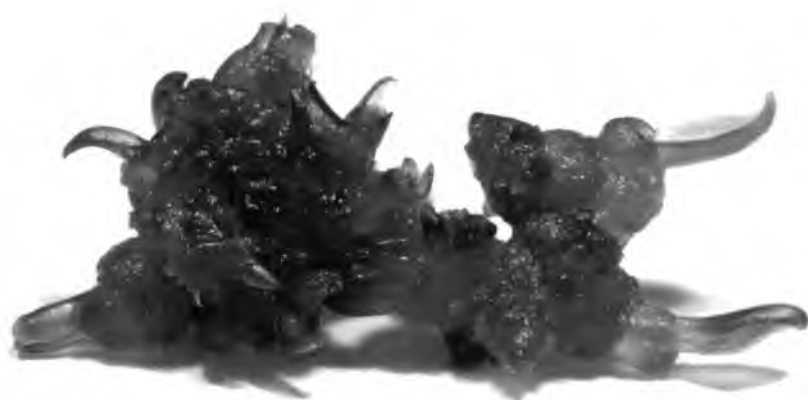


Fig. 18. Growth of protocorm-like bodies and plantlets on four different media (left to right) Vacin and Went with coconut water and sucrose, Vacin and Went with coconut water without sucrose, Vacin and Went with sucrose, Vacin and Went with neither coconut water nor sucrose. Protocorm-like bodies transferred from modified liquid Vacin and Went without sucrose (A x 1/3). Two to three leaf-stage protocorm-like bodies transferred from modified solid Vacin and Went without sucrose (B x 1/3). Plantlets transferred from solid Vacin and Went with sucrose (C x 1/3).

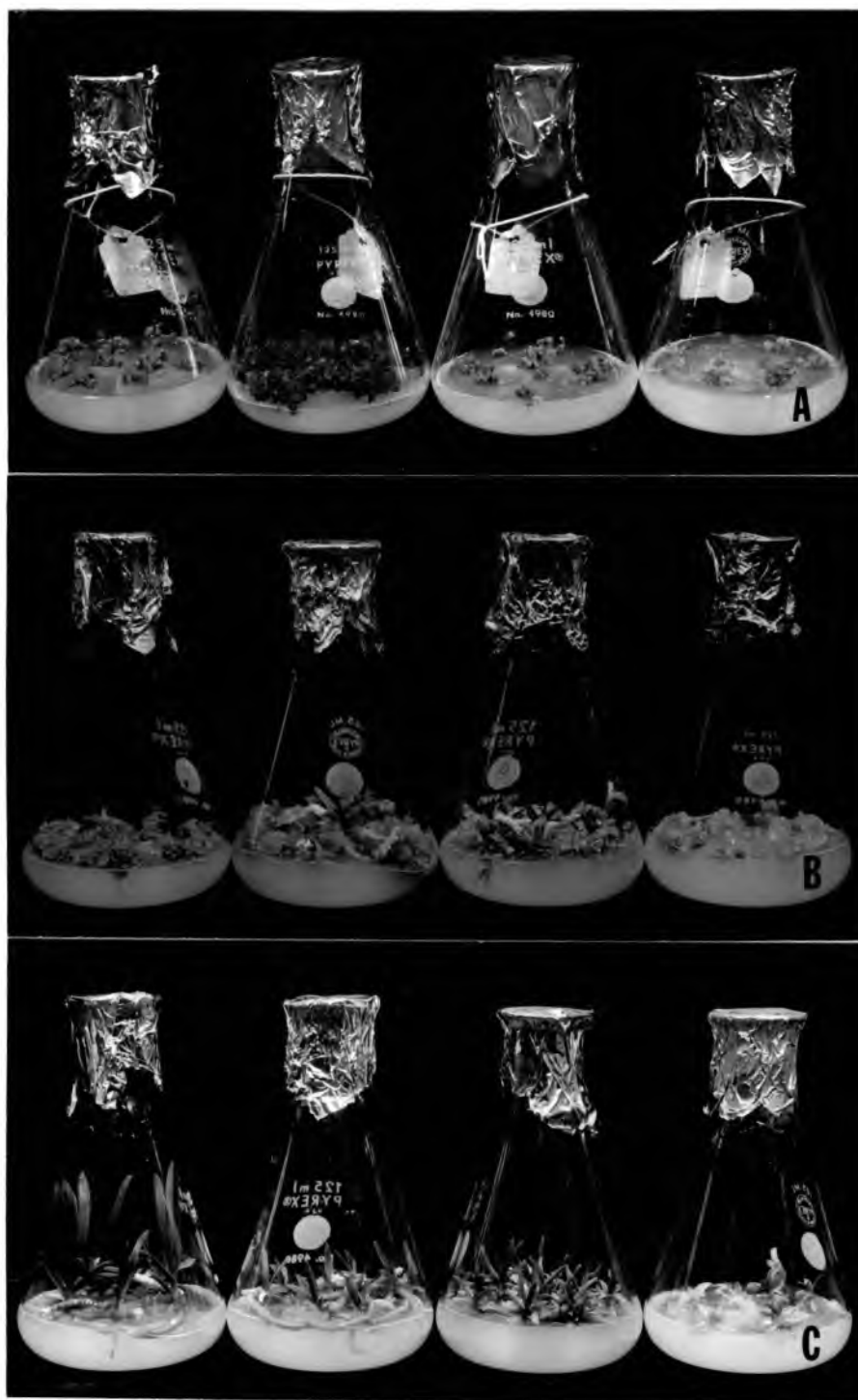
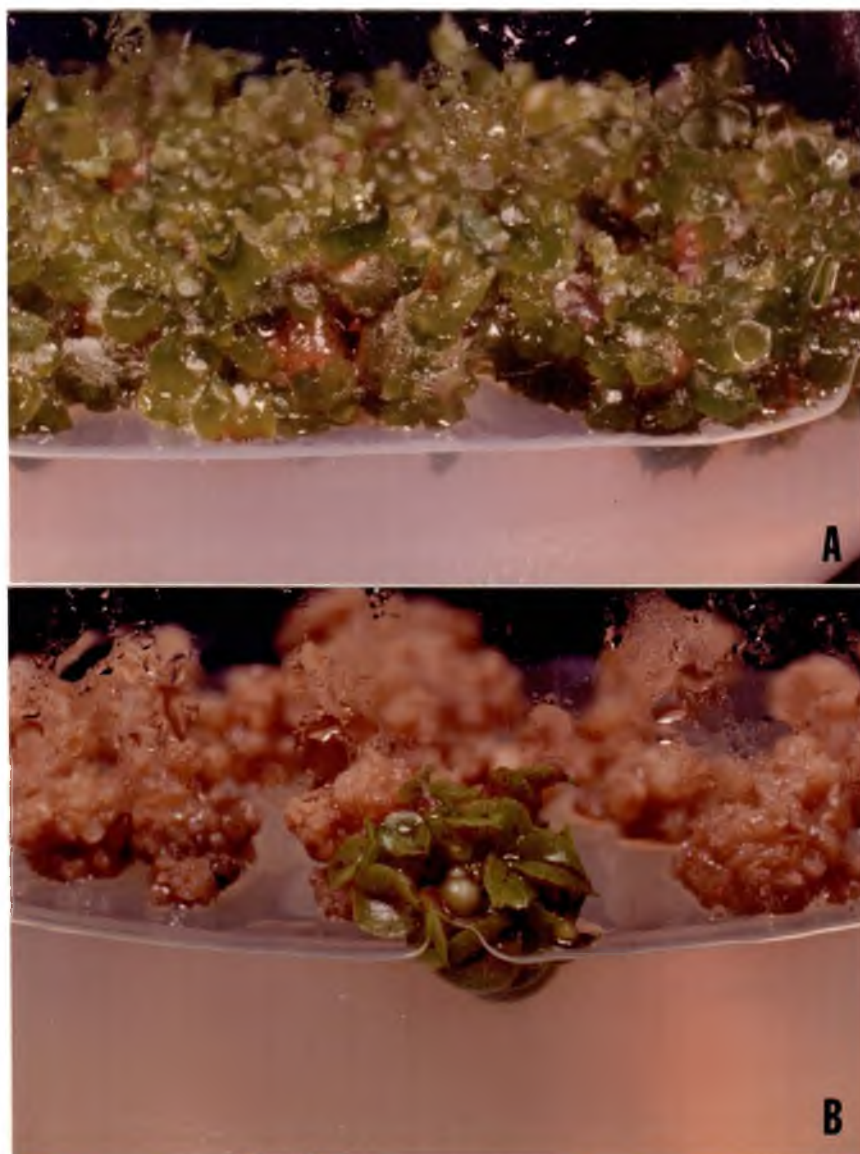


Fig. 19. Growth of protocorm-like bodies (plbs) on solid media.

Plbs on modified Vacin and Went without sucrose showed no roots but numerous rhizoids formed. Many plbs were produced (A x 5). Most plbs on Vacin and Went with sucrose turned brown but some plbs differentiated into plantlets (B x 5).



DISCUSSION

1. Study on the ability of different hybrids and different positions of explants to form plbs in culture

The use of apical buds for culture resulted in a high percentage of culture forming plbs (66.7%). Axillary buds from position one to four gave lower success (33.3 - 20.0%) while buds from position five and below did not proliferate at all. Morel (1971) pointed out that only juvenile cells have the regenerative properties to form plbs, mature cells did not grow. As buds get older (i.e., position of buds get further from the apex), there was a decrease in the number of explants which form plbs. Buds below position five were therefore not suitable for culture. This observation is contradictory to the report that axillary buds in Dendrobium showed an acropetal decreased in success of culture (Kim et al., 1970). Such difference may be due to the difference in growth habit of these two groups of plants. Dendrobium, sympodial orchid, may have less active buds acropetally, while the opposite is true of monopodial orchids.

Plants of different hybrids or within the same progeny vary in their response to meristem culture. Hybrids 1873 and 1800 were both Ascofinetia Twinkle, but the ability of cultures to form plbs were 66.7 - 25.0% respectively. Neostylis Lou Sneary 1907 gave the lowest percentage of success. Such differences in success may be due to genetic variations of the plant material. It might be of interest to work at the species level to see where such variations originated.

The use of floral buds and young inflorescence of dicotyledonous plants for culture have been reported by many workers (Konar and

Konar, 1966; Konar and Nataraja, 1969; Mohan Ram and Wadhi, 1968; Sehgal, 1968; and Ganapathy, 1969). The pedicel, torus, calyx (Konar and Nataraja, 1969), and inflorescence axis (Sehgal, 1968) proliferated and formed plantlets. Such proliferation was shown to originate from the epidermal cells all along the surface of the stem (Konar and Nataraja, 1969).

This study showed that the young inflorescence of a monocotyledon could also be induced to proliferate and form plantlets. Similarly, the epidermal cells all along the surface of the peduncle (Figs. 12, 13) showed meristematic activity, formed calli and eventually developed into plbs.

The use of young inflorescences for mericloneing monopodial orchids showed great potential, and also had the following advantages over the use of terminal and axillary buds:

- (1) the procedure is relatively easy,
- (2) contamination of cultures is lower,
- (3) percentage of cultures forming plbs is higher,
- (4) plbs can be obtained sooner than those from terminal and axillary buds. This is because the explant was cultured directly in liquid medium.
- (5) this method does not require sacrificing a plant, and there is no setback of parent plant through removal of young inflorescences.

2. Growth pattern of explants and differentiation into plantlets

The tissue from which plbs originate varies a great deal with genera and species. Morel (1971) reported that sometimes the outgrowth came from the central axis or leaf primordia as in Cymbidium, or

in Cattleya the axis remained inert and only the cells of the leaf epidermis proliferated. In the case of Vanda the cortex of the stem near the apex proliferated. In this study, the outgrowth came from the epidermis of the peduncle (Figs. 11, 12) as in the case of inflorescence culture. In the apical and axillary bud cultures, outgrowth originated from the cut surface of the scale-like leaves at the nodal area (Fig. 3A). Also in some cultures, young differentiated leaves proliferated (Fig. 3B).

The time required for tissues to proliferate and form plbs varies depending on types of tissues and species. In this study, the axillary bud and terminal buds took two to three months (Table I) to proliferate, while within one month, plbs were formed from the young inflorescence culture (Table II). Other workers reported that Cymbidium took one to two months (Morel, 1965) to produce plbs, while Cattleya and Dendrobium (Sagawa et al., 1966, 1967) took one and a half months.

The plbs formed from these cultures were very small compared to plbs from Cymbidium, Dendrobium and Cattleya. They did not form big clumps but separated by themselves while still on the shaker or on agar medium. For multiplication of plbs, no sectioning was necessary. In case of Cymbidium, Dendrobium and Cattleya, to encourage multiplication, the plbs have to be fractionated (Wimber, 1965).

Agitation of medium did not totally inhibit leaf differentiation of plbs (Fig. 6B). Leaves formed on plbs 75 - 100 days (Figs. 5, 15) after proliferation. As the flask became congested with the growing plbs, movement of tissue stopped. Two months after this stage, roots

appeared. From this, it is suggested that agitation of the medium affected root but not leaf differentiation. As long as plbs were agitated, roots did not form. If multiplication of plbs is desired, plb culture needs to be kept constantly agitated. Wimber (1963) reported that if cultures of Cymbidium were kept agitated, shoot formation was inhibited and only plbs formed. In miniature monopodial orchids studied, this was not observed; leaf differentiation occurred in agitated medium.

3. Media suitable for plbs growth into plantlets

Plbs proliferated well on media with coconut water but without sucrose. The presence of coconut water caused proliferation of tissues from the original explant, into callus, and also multiplication of plbs. Radley (1958) and Murashige (1961) suggested that gibberrellin, found in coconut water, inhibited organ differentiation. However, Reinert and Mohr (1967) reported that liquid medium with 1% coconut water caused death of Cattleya explants during the first three weeks of culture.

When plantlets were grown on medium containing coconut water and sugar, optimal growth was obtained (Fig. 16C). The stimulating effect of coconut water was accounted for by Radley (1958) to the supply of diverse metabolites found in coconut water which were required for growth.

Addition of 2% sucrose in the medium was found to be inhibitory to further multiplication of plbs. But sucrose was required for proliferation of the original explant tissue and also for root formation at a later stage. Lindemann (1970) showed that addition of

2% sucrose in medium caused Cattleya to form roots within ten days.

The ill effect of sucrose was not established. It is suggested that possibly the presence of sucrose in medium for multiplication of plbs of Sarcanthine orchids may be due to osmotic effects of the external medium or connected with chlorophyll degradation as evidenced by the chlorosis of the tissues.

For success and optimal growth of cultures, tissues had to be supplied with different media at various stages of development.

The following media were found most suitable:

(1) For proliferation of original explant tissue to callus, use liquid modified Vacin and Went.

(2) For multiplication of plbs, both solid or liquid modified Vacin and Went without sucrose media may be used.

(3) For maintaining plbs, use solid Vacin and Went without sucrose.

(4) For plbs differentiation into plantlets, use solid Vacin and Went.

(5) For good growth of plantlets, modified solid Vacin and Went is recommended.

This observation demonstrated the concept that nutrient and growth substance requirements of a given tissue vary depending on the physiological state, as evidenced by the morphological-anatomical differentiation of the explant. For proliferation, coconut water was required. Though the exact constituent of coconut water was not determined, many workers (van Overbeek, 1941; Caplin and Steward, 1948) have reported that it contained various growth promoters,

principally kinetins (van Overbeek, 1941) which were known to cause cell division. The differentiation of roots from plbs required the addition of sucrose. In this way, growth and development of tissues could be manipulated by altering the components of the growth media.

SUMMARY

The miniature monopodial orchids (Ascofinetia Twinkle, Ascofinetia Cherry Blossum, Vandofinetia Premier, Neostylis Lou Sneary, Vascostylis Blue Fairy) were successfully mericlone on modified Vacin and Went medium. Explants from the young inflorescence were the most suitable material for culture, though apical buds and axillary buds near the apex could also be used. In the terminal and axillary bud culture, plbs originated from the cut surface of the scale-like leaves at the nodal area or from tissue of the young leaves. In inflorescence culture, plbs were derived from the epidermal cells. Growth of plbs was initially slow but increased rapidly with time. Three explant development patterns were observed: explant grew directly into plantlets, produced both shoots and plbs, or produced plbs only. Anatomical study revealed plbs consisted of undifferentiated mass of parenchymatous cells with areas of meristematic activity from which apical meristem and leaves originated. Vascular tissue was observed after leaves differentiated on the plbs.

Addition of coconut water resulted in proliferation of tissues, while addition of sucrose resulted in necrosis of the undifferentiated plbs. Sucrose was required for root formation. For differentiation of plbs into plantlets and optimal growth of plantlets, solid Vacin and Went and solid modified Vacin and Went were found to be most suitable.

This study demonstrated that monopodial miniature orchids did respond to meristem culture. Propagation of this group of orchids is not difficult, and has a similar commercial potential as in Cymbidium and Dendrobium.

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